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Roles for Zebrafish Focal Adhesion Kinase in Notochord and Somite Morphogenesis

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We have cloned zebrafish focal adhesion kinase (Fak) and analyzed its subcellular localization. Fak protein is localized at the cortex of notochord cells and at the notochord–somite boundary. During somitogenesis, Fak protein becomes concentrated at the basal region of epithelial cells at intersomitic boundaries. Phosphorylated Fak protein is seen at both the notochord–somite boundary and intersomitic boundaries, consistent with a role for Fak in boundary formation and maintenance. The localization of Fak protein to the basal region of epithelial cells in *knypek;trilobite* double mutant embryos shows that polarization of Fak distribution in the somite border cells is independent of internal mesenchymal cells. In addition, we show that neither Notch signaling through *Suppressor of Hairless* (*SuH*) nor *deltaD* is necessary for the wild-type segmental pattern of *fak* mRNA expression in the anterior paraxial mesoderm. However, nonsegmental expression of *fak* mRNA occurs with ectopic activation of Notch signaling through *SuH* and also in *fused somite* and *beamter* mutant embryos, indicating that there are multiple regulators of *fak* mRNA expression. Our results suggest that Fak plays a central role in notochord and somite morphogenesis. © 2001 Elsevier Science

Key Words: **focal adhesion kinase; phosphorylation; zebrafish; notochord; somitogenesis; Notch; *knypek*; *trilobite*; *fused-somite*.**

INTRODUCTION

Embryonic morphogenesis is a result of coordinated cell movements and cell-shape changes. Although much is known about intracellular signaling mechanisms underlying cell motility in tissue culture cells (Cary *et al.*, 1998; Nobes and Hall, 1995), less is known about the signaling pathways that modulate cell migration, adhesion, and shape changes during embryonic morphogenesis (Ridyard and Sanders, 1999). Cell motility requires a cycle of membrane extension, adhesion to the extracellular matrix (ECM),[3] cell body translocation, and finally deadhesion from the ECM (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). One intracellular constituent of focal complexes that anchors the actin cytoskeleton to the ECM is focal adhesion kinase (FAK), a nonreceptor tyrosine kinase. FAK signaling plays crucial roles in cell migration in cultured cells as well as in developing tissues and embryos (Gilmore and Romer, 1996; Ilic *et al.*, 1995; Ridyard and Sanders, 1999).

FAK has been implicated in promoting both cell motility and cell adhesion. The phosphorylation state of FAK determines whether FAK acts to promote cell migration or cell adhesion (Burridge *et al.*, 1992; Yu *et al.*, 1998). High levels of phosphorylation promote adhesion and moderate levels of phosphorylation with phosphorylation turnover promote migration. In addition, $FAK^{-/-}$ null mouse embryos initiate gastrulation; however, no notochord or somites form (Furuta *et al.*, 1995; Ilic *et al.*, 1995). Cells isolated from $FAK^{-/-}$ mouse embryos exhibit reduced motility and increased

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[3] ECM stands for extracellular matrix. Throughout this article, we use italics to designate the mRNA product of the zebrafish focal adhesion kinase (FAK) gene: *fak* thus refers to focal adhesion kinase mRNA and the *fak* gene. Fak designates zebrafish focal adhesion kinase protein. FAK refers to mouse, *Xenopus*, or human focal adhesion kinase in accordance with that literature.

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focal adhesions compared to wild-type cells (Ilic *et al.*, 1995, 1996). These observations support the hypothesis that FAK promotes cell motility by increasing the turnover of focal adhesion complexes. It has also been postulated that FAK, in some contexts, may function to stabilize tissue boundaries. For instance, FAK is concentrated at the myotendinous junction in *Xenopus* muscle fibers and may play a role in stabilization of this junction (Baker *et al.*, 1994). In addition, FAK protein also localizes to somite boundaries in both *Xenopus* and mouse embryos (Hens and DeSimone, 1995; Polte *et al.*, 1994). The localization of FAK at stable tissue boundaries suggests a role for FAK in the formation and/or maintenance of these boundaries.

Because of the potential regulatory role of focal adhesion kinase in morphogenesis, we isolated zebrafish *fak* cDNA and analyzed the subcellular developmental expression of *fak* mRNA, Fak protein, and the phosphorylation state of Fak_{tyr397}. Low levels of Fak protein are observed in all cells throughout development. Interestingly, although the cell behaviors that underlie notochord and somite formation are distinctly different, we found that *fak* mRNA and Fak protein are expressed at high levels in both the forming notochord and somites. The time course of *fak* mRNA and Fak protein expression in the notochord correlates with the anterior–posterior progression of intercalation in the notochord. This expression suggests a role for Fak in modulating notochord cell intercalation. During somite formation, Fak protein becomes concentrated at the intersomitic boundary, suggesting a role for Fak in the formation and/or stabilization of somite borders. We tested whether *fak* mRNA expression in the presomitic mesoderm is dependent on Notch signaling through *suppressor of hairless* as is *paraxial protocadherin* (Kim *et al.*, 2000). Interestingly, disruption of somitogenesis via inhibition of Notch signaling through *suppressor of hairless* does not affect *fak* mRNA expression. We also asked whether *fak* mRNA was normally expressed in the *fused somite*-type mutants, which do not form more posterior somites. *fak* expression is affected in two different ways in each of the *fused somite-type* mutants (*after eight/DeltaD*, *fused somites*, *beamter*, *deadly seven*), indicating that *fak* expression in the paraxial mesoderm is exquisitely sensitive to the local environment. The subcellular localization and high expression of Fak protein in notochord and somites indicates roles for Fak in notogenesis and somitogenesis.

MATERIALS AND METHODS

Maintenance of Fish

Breeding fish were maintained between 24.5 and 26.5°C on a 14-h light/10-h dark cycle. Natural spawning was used to collect embryos that were staged according to Kimmel *et al.* (1995). The recessive mutants *knypek*^{m119}*;trilobite*^{m209} (*kny;tri*) were from Vanderbilt University stocks, *no tail*^{b195} (*ntl*), *spadetail*^{b104} (*spt*), and *floating head*^{n1} (*flh*) were from the University of Oregon stocks (Halpern *et al.*, 1993; Kimmel *et al.*, 1989; Marlow *et al.*, 1998; Talbot *et al.*, 1995), and *fused somites*^{te314a} (*fss*), *beamter*^{to202} (*bea*), *deadly seven*^{tx201}(*des*), and *after eight*^{tr233}(*aei*) (van Eeden *et al.*, 1996) embryos were generously provided by Andrew C. Oates.

Cloning of fak cDNA

A PCR-based strategy was used to isolate a probe for zebrafish *fak*. The probe was a 566-bp DNA fragment amplified from a zebrafish gastrula-stage cDNA library (provided by Dr. Randall T. Moon) using forward degenerate primer 5′-GAA/G GAA/G GAC/T ACI TAC/T ACI ATG CCI-3′ (I is 2′-deoxyInosine) and reverse degenerate primer 5′-GGI GCC ATC CAC/T TTA/G/T ATI GG-3′ from the kinase domain of *fak*. *fak* cDNA was isolated from a zebrafish gastrula-stage cDNA library provided by Drs. Thiery LePage and David Kimelman. Subsequently, the ESTs AI958105 fc90g10.x1 and AI958105 fc90g10.y1 were submitted to GenBank with less than 1% difference between their sequences and our clones. This EST most probably represents the transcript from *fak*, the few sequence differences probably due to either polymorphisms or sequencing artifacts of the EST. The GenBank accession number for the full-length zebrafish *fak* sequence is AAK31154.

Mapping of fak

For meiotic mapping of *fak*, we identified genetic polymorphisms segregating in the HS meiotic mapping panel as described by Kelly *et al.* (2000) and Woods *et al.* (2000) and double-checked the location in the LN54 radiation hybrid panel as described by Hukriede *et al.* (1999). Genomic DNAs from the mapping panels were amplified by using primers for the 3′-untranslated region of *fak* (forward *fak* +128 GCCGGGCTCTGGATTTATTTA, reverse *fak* −437 CAGTCCTAGGAGAAGCGTGAGAGT), giving a 310-bp fragment. Comparative mapping was accomplished as described by Woods *et al.* (2000).

Western Blot Analysis

Sixty embryos were dechorionated and their cells dissociated at 4°C by triturating with a 200-μl pipetman in 0.3 ml PBS containing 1 mM EDTA, 0.3 mM PMSF, 0.1 mg/ml soybean trypsin inhibitor (Sigma), and 70 μg/ml leupeptin and aprotinin (ICM). The cells from early embryos were gently centrifuged at 4°C and 500–1000 rpm in a microfuge for 1 min. Cells from later stages were centrifuged at up to 2000 rpm. Centrifugation was repeated after turning the tubes 180° to pellet cells adhering to the sidewalls of the microfuge tube. The supernatant was removed and all cells resuspended and recentrifuged once or twice in 0.3 ml of the protease inhibitor cocktail to remove all soluble yolk proteins while preventing degradation of cellular proteins. Cells were pelleted and 60 μl of SDS–PAGE buffer was added to each sample making the final extract the equivalent of 1 embryo/μl and stored at −80°C. Novex 8% Tris–Glycine precast gels were used and 10 μl (the equivalent of 10 embryos) was added per gel lane. Gels were transferred to nitrocellulose, probed with a 1:5000 dilution of primary antibody (FAK C-20; Santa Cruz Biotechnology), and detected by using ECL+ Kit (Amersham). The specificity of C-20 for zebrafish Fak was verified by expressing the zebrafish *fak* cDNA using a PROTEINscript II combined *in vitro* transcription and translation kit (Ambion, Inc.), and probing Western blots of the translations with C-20.

For densitometric determinations of the relative abundance of Fak and phosphorylated Fak during development, a similar proce-

dure was used. However, embryos were dissociated in embryo medium (Westerfield, 1993) with double-strength protease inhibitor cocktail (Roche) and 10 mM sodium metavanadate to inhibit artifactual dephosphorylation of Fak during lysate preparation. Ten microliters of lysate were loaded in each lane of a 10% precast gel. Nitrocellulose blots were probed with affinity-purified anti-pY397Fak (BioSource) at 1:2500. Blots were then stripped using "Restore" Western blot stripping buffer (Pierce), washed, and reprobed with anti-Fak as above. Films were scanned at 300 dpi (8 bits per pixel) by using a UMAX transparency scanner, and densitometry was preformed by using Multi-Analyst (Bio-Rad). We calculated relative signal strength by setting the strongest signal from each blot to 1 and determining all other signals with respect to this value.

Whole-Mount in Situ Hybridization and Antibody Staining

Single and double whole-mount *in situ* hybridization was with an antisense *in situ* probe that hybridized with the 3′-untranslated region (Jowett, 1999). Sense controls showed no specific staining (data not shown).

Whole-mount immunostaining was used to determine the subcellular localization of Fak protein. Embryos were dechorinated and fixed with 2% TCA (Aldrich) in PBS for 1–10 h at 4°C, rinsed over 2 h with PBDT (1× PBS, 1% BSA, 1% DMSO, 0.1% Triton-X 100, pH 7.3), and then blocked in PBDT with 2% goat serum at room temperature for 2 h. Primary antibody to FAK was added at a 1:100 dilution (C-20; Santa Cruz Biotechnology) and incubated overnight at 4°C. Embryos were washed for 2 h at room temperature in PBDT, and the secondary (goat anti-mouse Alexa 488; Molecular Probes) was added at a 1:200 dilution and incubated either 4 h at room temperature or overnight at 4°C. Immunostaining with anti-pY397Fak was preformed similarly with the exception that embryos were fixed overnight at 4°C in 4% paraformaldehyde.

Microscopy, Vital Staining, and Time-Lapse Analysis

Embryo morphology and *in situs* were viewed by using an upright Nikon microscope fitted for Hoffman modulation contrast optics. Pictures were obtained with a Nikon CoolPix 950 digital camera. Immunofluorescence data were obtained by using either a BioRad MRC-600 confocal microscope or a BioRad Radiance 2000 confocal microscope using 20× 0.4 NA and 60× 1.3 NA objectives. The protocol of Beck *et al.* (2000) was used to photomontage high-magnification images of embryos in order to expand the field of view and accommodate the depth of field limitations of the optics used (Beck *et al.*, 2000).

Live embryos were stained with Bodipy-Ceramide (Molecular Probes) and images were captured according to the protocol of (Cooper *et al.*, 1999). For time-lapse analysis, individual images were compiled into time-lapse movies by using NIH Image and 4 d Turnaround (both programs are freely available on the web).

Injection of Suppressor of Hairless Constructs

Embryos were injected at the 2- to 4-cell stage with 0.5 nl of 400 ng/μl *Su(H)* mRNA. mRNA was made by using Ambion Message Machine. Control embryos were injected with 0.5 nl 400 ng/μl GFP mRNA. In some experiments (at least 2 experiments for each construct), embryos were coinjected with both *Su(H)* mRNA and GFP mRNA and fluorescence was seen only on the injected side of the embryo. Thus, one-half of the embryo serves as an internal control against which to compare the other half of the embryo. Embryos injected with the *Su(H)* constructs were collected at the 10-somite stage, and those embryos with defects in somite formation on one side of the notochord were photographed and fixed for single embryo *in situ* hybridization ($n = 4$ or 5 experiments for each construct). Control embryos injected with GFP were also collected and fixed at the 10-somite stage, and none of these embryos ($n = 80$) showed defects in *fak* expression. *In situ* hybridization was performed as described above.

RESULTS

Cloning, Mapping, and Western Analysis of fak

One full-length cDNA and three partial cDNAs of the zebrafish *fak* gene were recovered from a gastrula-stage library. The full-length clone of Zebrafish *fak* is 3913 bp and contains a complete open reading frame encoding 1049 amino acids (Fig. 1). Eighty-one percent of the amino acids are identical with chick and human focal adhesion kinase. Importantly, many functional regions are conserved, including the kinase domain (thr-414 to leu-678, 94% identical); the focal adhesion-targeting domain (gln-899 to leu-1040, 97% identical); 26 of the 27 tyrosine residues including tyrosine-397 (position 399 in the zebrafish sequence), which is autophosphorylated and binds *src.* This site is necessary for FAK-stimulated cell migration (Cary *et al.*, 1996). In addition, tyr-925 (position 923 in the zebrafish sequence), which is a site for Grb2 binding and is necessary for the anti-apoptotic activity of FAK, is conserved (Schlaepfer *et al.*, 1994; Sonoda *et al.*, 2000). Finally the proline-rich region (pro-714 to Ser 720) that binds *cas* and is involved in regulating cell migration is also conserved (Cary *et al.*, 1998).

Zebrafish *fak* maps to linkage group 16 (LG16) (Fig. 2A) with high statistical significance (to AA549805 with LOD 8.3, to AI476862 with LOD 9.9). Analysis of apparent orthologues shows that LG16 shares syntenies with the distal tip of human chromosome 8, and with the 14-cM interval on mouse chromosome 15 (Fig. 2A). As shown recently, during vertebrate evolution, intrachromosomal rearrangements, including inversions, frequently alter locus order without disrupting conserved syntenies (Postlethwait *et al.*, 2000). These comparative mapping data support the assignment of zebrafish *fak* as an orthologue of human *PTK2/FAK/FADK* and mouse *Fadk*, and show that this chromosome region has remained syntenic since the zebrafish and human lineages diverged in the late Ordovician (450 million years ago) (Kumar and Hedges, 1998).

We used Western blots to determine when Fak protein is expressed during development. Several lines of evidence indicate that a commercially available antibody (C-20; Santa Cruz Technology) recognizes Zebrafish Fak. First, C-20 recognizes a single band of the correct size on a Western blot (Fig. 2). Second, the putative Fak protein is

FIG. 1. Zebrafish focal adhesion kinase (Fak) sequence is conserved in the kinase domain, the focal adhesion-targeting domain (FAT), most tyrosine residues, and the proline-rich region that binds *Cas*. Primary structure of zebrafish (*Danio rerio*, Dre) focal adhesion kinase compared with human (HUM), and chick (Galina, Gga) FAK (accession numbers AAK31154, Q05397, and Q00944, respectively).

strongly expressed in the organ rudiments that strongly express *fak* mRNA. Also, in *spadetail* and *no tail* zebrafish embryos, Fak protein is only strongly expressed in the organ rudiments where *fak* mRNA expression is unaffected by these mutations (data not shown). In addition, an antibody raised against the phosphorylated peptide sequence surrounding tyrosine 397 of the human Fak protein and zebrafish protein recognizes the same band as C-20 on blots. Finally, when our zebrafish fak cDNA is transcribed and translated *in vitro*, the protein product is recognized on Western blots probed with C-20 and runs at the same relative mobility as the band detected by C-20 in embryo lysates (data not shown).

Fak protein occurs as early as the 256-cell stage before the midblastula transition, indicating that either *fak* mRNA or Fak protein is maternally supplied (Fig. 2B). Fak protein is present throughout development and is slightly increasing in abundance after the onset of segmentation.

Relative Abundance of Fak and Phosphorylated Fak during Development

Although Fak is present throughout development, its relative abundance and phosphorylation state are dynamic. We quantified these changes using densitometry of immunoblots probed with anti-pY397Fak and anti-Fak sequentially (Fig. 3). These data show that Fak protein is present at modest levels during cleavage and gastrulation (Fig. 3, open bars), but is not phosphorylated at detectable levels until very near the end of gastrulation, at tail-bud stage (10 h at 28.5°C; Fig. 3, dotted bars). At the beginning of somitogenesis, the relative abundance of Fak protein increases modestly, and Fak is moderately phosphorylated. Finally, during the pharyngula period of development (prim-5 through prim-12), Fak remains relatively abundant, but phosphorylation levels increase dramatically. Examination of the ratio

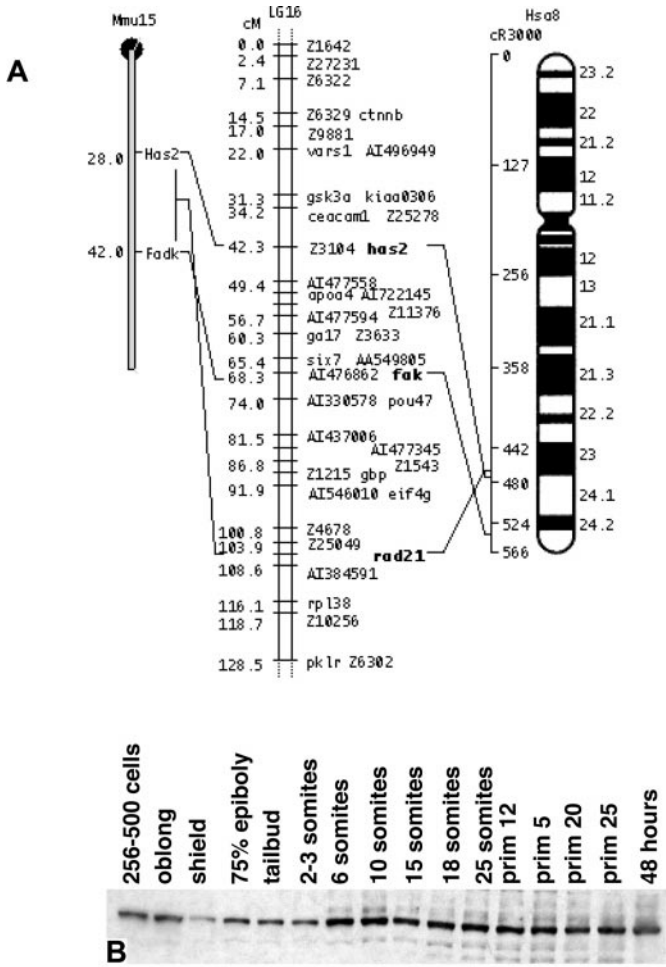

FIG. 2. Conserved syntenies support the conclusion that *fak* and *PTK2* are orthologues. Three loci on LG 16 have apparent orthologues on human (Hsa) chromosome 8q and mouse (Mmu) chromosome 15. These three loci are (in order, fish/human/mouse): *fak*,*/PTK2/Fadk*; *rad21(AI477810)/RAD21/Rad21*; *has2(U53223)/HAS2/Has2*. Other named loci in the figure are apparent orthologues of loci on other human chromosomes (Woods *et al.*, 2000). It is interesting to note that while *PTK2/FAK/FADK* is at *Has 8q24-qter*, the closely related gene *PTK2B/CAKβ* is distantly located on the same chromosome at *Has 8p21.* (B) Fak protein is expressed throughout early development and expression increases slightly during somitogenesis. Ten embryos were added per lane.

of the relative phosphorylation of Fak to the relative abundance of Fak (Fig. 3, crosshatched bars) reveals three distinct phases: a phase during which Fak is unphosphorylated during cleavage and gastrulation, a phase of modest phosphorylation (or high phosphorylation turnover) during segmentation, and a phase of high phosphorylation subsequent to segmentation.

Fak May Play a Role in Notogenesis

The first organ primordium to express high levels of *fak* mRNA is the developing notochord at the tailbud to 1-somite stage (Fig. 4A). *fak* is detected in all intercalating notochord cells (Fig. 4F). Expression of *fak* fades from the anterior notochord once this region has undergone convergence (Fig. 4D red arrow), but *fak* continues to be expressed in the posterior region of the notochord that is still undergoing cellular intercalation. Thus, *fak* mRNA expression coincides with the anterior-to-posterior progression of convergence and is highest in the portion of the notochord that is undergoing the final phase of intercalation, which is the narrowing of the notochord from two to three cells wide to one cell wide.

Time-lapse confocal microscopy indicates that, in zebrafish embryos, as in *Xenopus* embryos (Keller *et al.*, 1989), intercalating notochord cells extend protrusions that insert between adjacent cells and then extend to the notochord-somite boundary (Figs. 5A and 5B). As the notochord intercalates from two cells wide to one cell wide, cells extend a thin protrusion between adjacent cells (Figs. 5C and 5D). Next, these protrusions are extended to the opposite side of the notochord (Fig. 5E). Finally, these protrusions gradually increase in volume as the whole cell narrows in the anterior–posterior dimension (Figs. 5F–5H).

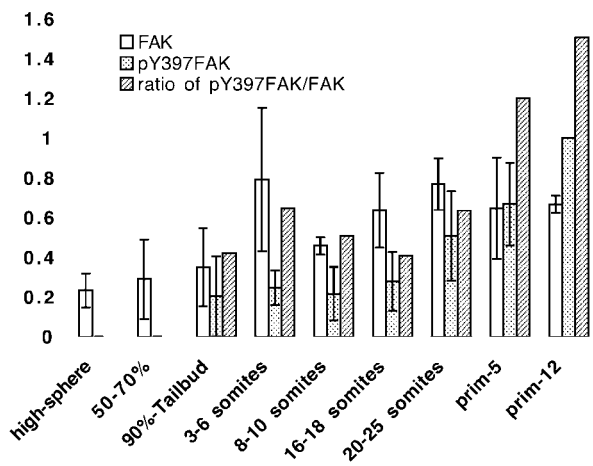

FIG. 3. Densitometric quantification of immunoblots of embryo lysates probed with anti-pY397Fak then with anti-Fak sequentially show that Fak protein is not phosphorylated until tailbud stage, is moderately phosphorylated during somitogenesis, and is highly phosphorylated thereafter. High-sphere-stage embryos (3–4 h at 28.5°C). 50–70% epiboly-stage embryos (5–7 h). 90% epiboly to tail bud stage embryos (9–10 h). 3- to 6-somite embryos (11–12 h). 8- to 10-somite embryos (13–14 h). 16- to 18-somite stage embryos (17–18 h). 20- to 25-somite embryos (19–21 h). Primordium-5-stage embryos (23–25 h). Primordium-12-stage embryos (27–30 h). Open bars, Blots stained with anti-FAK. Dotted bars, Blots stained with anti-pY^{397} FAK. Stripped bars, Ratio of relative pY^{397}Fak signal to relative Fak signal.

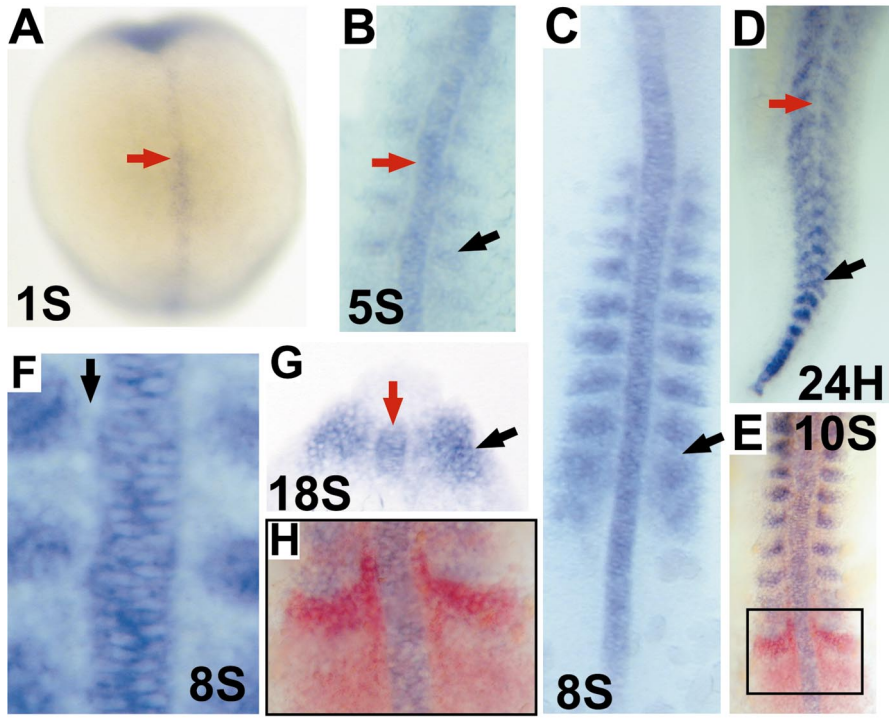

FIG. 4. *fak* mRNA is expressed in the notochord, somite, and unsegmented paraxial mesoderm in wild-type zebrafish embryos. (A–F, H) Dorsal view. (G) Transverse view. "S" indicates somite stages. (A, B) Expression of *fak* in the notochord (red arrows) and somites (black arrows) in early embryos. (C) The black arrow points to the posterior border of the eighth (last formed) somite. (D) Expression of *fak* in somites (black arrow) in a late embryo. The absence of notochordal *fak* is indicated at the red arrow. (E, H) Two-color *in situ* hybridization (H is enlarged view of the box region in E) of the localization of *fak* (blue) and the two strong bands of *papc* expression (red) in the unsegmented paraxial mesoderm. (F) *fak* is not expressed in the adaxial cells that abut the notochord (black arrow). (G) A cross-section of an 18-somite embryo indicates that *fak* is expressed throughout the region of the notochord that is still undergoing cell intercalation (red arrow) and throughout the dorsal–ventral extent of the somites (black arrow).

Therefore, notochord formation in zebrafish embryos, as in *Xenopus* embryos, is a result of stereotypical cell intercalation behavior.

We used the C-20 antibody to human FAK to determine whether the time course of Fak protein expression in the zebrafish notochord also correlates with the intercalation of notochord cells. Although Fak localizes to the entire cortex of intercalating notochord cells (Figs. 5J and 5K), there are frequently subcellular domains of increased concentrations of Fak protein that we call plaques of Fak (Fig. 5L). The plaques appear to be located at the overlapping edges of notochord cells (white arrow, Fig. 5L). Fak also localizes to the region of the notochord–somite boundary (Figs. 5I–5K), suggesting a potential role for Fak in adherence of intercalating cells to this boundary. As predicted, Fak protein expression is highest in the portion of the notochord undergoing the final phase of convergence (Figs. 5I and 5J, arrowheads), and thus, reflects the anterior–posterior progression of intercalating activity of notochord cells.

FAK protein becomes phosphorylated upon adhesion to the extracellular matrix (Burridge *et al.*, 1992). Phosphorylation occurs on several sites of FAK in response to integrin stimulation, but the primary autophosphorylation site is Tyr-397 (Calalb *et al.*, 1995; Schlaepfer and Hunter, 1996). This site also is necessary for FAK to rescue migration of $FAK^{-/-}$ cells on fibronectin (Sieg *et al.*, 1999). Although it has been demonstrated that phophotyrosine-containing FAK is present during neurulation in *Xenopus* (Hens and DeSimone, 1995), the spatial distribution of such phosphorylated FAK has not been examined. Therefore, we asked whether the Fak present in the intercalating notochord cells of the fish embryo is phosphorylated. Phosphorylated Fak is clearly present at the notochord periphery (Figs. 5M and 5N). Phosphorylated Fak appears to be present in subcellular striations that encircle the notochord perpendicular to the anterior–posterior axis (Fig. 5N). Thus, the localization of phosphorylated Fak suggests that it is activated where the cells of the notochord make adhesive contacts with the ECM surrounding the notochord. In order to investigate the possibility that rapid turnover of phosphorylation of Fak was occurring, we incubated embryos in embryo medium with 10 mM vanadate for 0, 1, and 2 h before fixation and

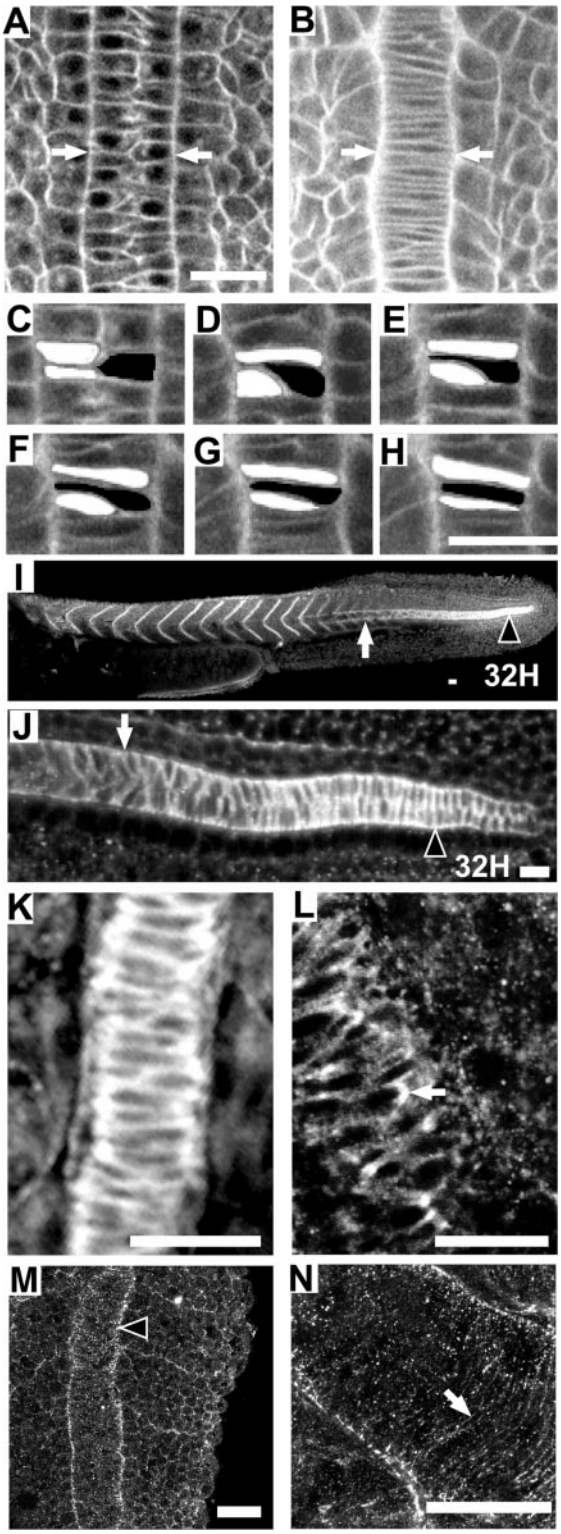

FIG. 5. Zebrafish notochord cells intercalate as in *Xenopus* embryos; Fak protein is highly expressed in the region of the notochord that is undergoing convergence; and Fak is phosphorylated at the notochord-somite boundary. Anterior is towards the top or left in all panels. Dorsal views except (I) and (J), which are side views. (A–H) Time-lapse analysis of a vitally-stained zebrafish embryo (see methods) from the 1-somite stage through the 4-somite stage at the level of the first 4 somites. At time 0 min (A), the notochord is two cells wide (white arrows) and by time 38 min (B) cellular intercalation results in a notochord that is one cell wide. (C–H) Enlarged view of three cells intercalating. (C) 0 min. (D) 10 min. (E) 23 min. (F) 30 min. (G) 35 min. (H) 40 min. (I–L) Immunostaining for Fak protein. The posterior region of the notochord undergoing cell intercalation is noted with an arrowhead. White arrow indicates the notochord–somite boundary anterior to the intercalating domain. (K) A 10-somite embryo, dorsal view at the level of somite 4 showing the notochord–somite boundary. (L) Enlarged view of a notochord undergoing intercalation in a 10-somite embryo, at the level of somite 8. An arrow notes a discrete plaque of Fak. (M, N) Immunostaining for pY^{397}FAK. M: A 13-somite embryo at somite 13 stained for phospho-Fak at the notochord-somite boundary (black arrowhead). (N) Projection of confocal sections that include the entire thickness of notochord of a 13-somite embryo showing circumferential striations in phospho-Fak staining perpendicular to the notochord axis. Scale bars, 20 μm, with the exception of (I), where scale bar is 40 μm.

immunostaining with anti-pY397Fak. Although we observed an increase in general staining intensity following incubations in vanadate, no specific changes in the spatial pattern of staining were observed (data not shown). If Fak is phosphorylated at sites of cell–cell intercalation in the notochord, it is at a level that we were unable to detect in these assays. Clearly, the localization of detectable phosphorylated Fak indicates a role for Fak in adhesion to the sheath of ECM surrounding the notochord during its morphogenesis.

Fak May Play a Role in Somitogenesis

During somite formation, a sheet of mesenchymal cells, the paraxial mesoderm, is transformed into a central aggregate of mesenchymal cells surrounded by an epithelial layer (Gossler and Hrabe de Angelis, 1998). It has been proposed that cell–matrix interactions play a role in somite morphogenesis in chicks (Duband *et al.*, 1987; Lash *et al.*, 1987). We asked whether *fak* mRNA and protein are expressed in forming somites. *fak* mRNA is faintly and diffusely expressed in the paraxial mesoderm at the 1-somite stage (Fig. 4A), but by the 5-somite stage *fak* is expressed in stripes in the posterior half of somites (Fig. 4B) and weakly expressed in the presomitic mesoderm. These stripes become intensified by the 8-somite stage (Fig. 4C), at which point *fak* is also strongly expressed in the next forming somites, S0 and S-1, albeit less segmentally than in formed somites. The arrow in Fig. 4C denotes the posterior-most somite boundary and by convention, S0 designates the forming somite, and S-1 the next posterior presumptive somite (Christ and Ordahl, 1995). Hoffman optics was used to visualize intersomitic furrows. In addition, two-color *in situ* hybridization

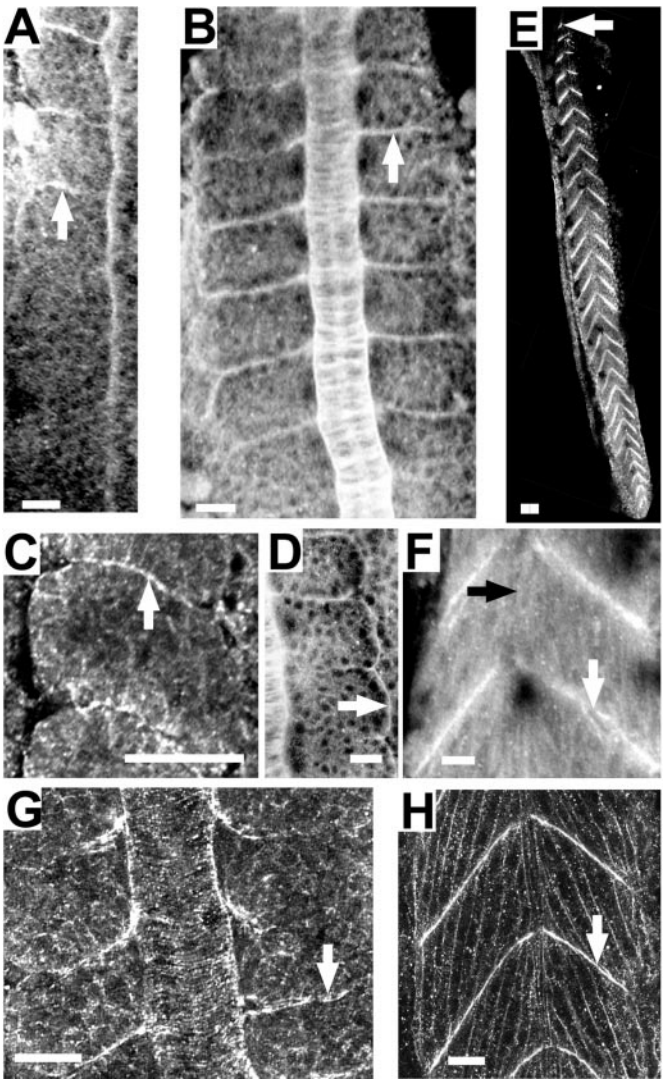

FIG. 6. Fak protein and phosphorylated Fak become concentrated at somite boundaries during somitogenesis and persist throughout myotome development. (A–D, G) Dorsal views. (E, F, H) Side views. (A–F) Fak stained with anti-human Fak. (G, H) Phospho-Fak stained with anti-pY397FAK. (A) 10-somite embryo (white arrow designates last formed somite). (B) 7-somite embryo. White arrow designates a somite boundary. (C) A higher magnification of Fak in a 10-somite embryo. Arrow indicates the basal side of border cells that abut the intersomitic furrow (arrow). (D) 7-somite embryo. Arrow indicates the lateral edges of the lateral epithelial cells of a somite. (E, F) 32-h embryos. (G) 10-somite embryo. (H) 30-h embryo. Scale bars, 20 μm, with the exception of (E), where scale bar is 40 μm.

for *fak* and *paraxial protocadherin* (*papc*) was used to confirm *fak* expression in the posterior half of somites and in the presomitic mesoderm since *papc* is expressed in two strong bands in the anterior halves of S0 and S-1 (Yamamoto *et al.*, 1998). Expression of *fak* colocalized with these bands of *papc* in the unsegmented paraxial mesoderm as well as with the posterior half of the last-formed somite (Figs. 4E and 4H). Thus, the segmental expression of *fak* in formed somites indicates that either *fak* expression becomes stronger in the posterior half of a somite or is slightly downregulated in the anterior half of a somite to generate a segmental pattern (Figs. 4C and 4E). Unlike *papc* expression, *fak* mRNA expression in formed somites is persistent: *fak* mRNA was typically seen in at least the 10–15 most recently formed somites. In tail somites, the exact zone of highest *fak* expression is slightly variable: *fak* is sometimes stronger in the center rather than the posterior of a somite (Fig. 4D). Interestingly, *fak* expression in the adaxial cells that abut the notochord is markedly less than in the lateral somite cells (Figs. 4C and 4F). By 32 h of development, *fak* mRNA is not detected in the somites and is expressed only in the posterior-most region of the notochord undergoing convergence (data not shown). The expression of *fak* in forming and recently formed somites indicates a potential role for Fak protein in the cell behaviors that underlie the formation of a somite boundary.

Examination of the subcellular localization of Fak protein during somitogenesis also indicates a role for Fak in somite boundary formation. Fak protein is not subcellularly localized in the presomitic mesoderm (lower half of Fig. 6A). However, during somite formation, Fak becomes concentrated in the basal region of epitheloid border cells that abut

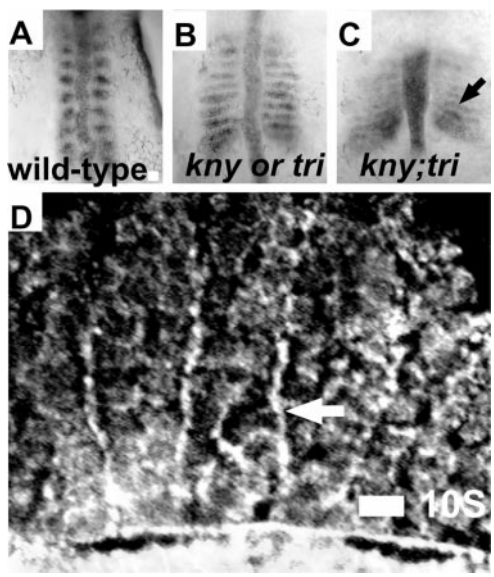

FIG. 7. Fak protein localizes to somite boundaries in *kny;tri* double-mutant embryos that have somites comprised of only two rows of epithelial border cells. (A–C) *In situs* for *fak* mRNA expression in 9-somite embryos (dorsal views with anterior up). (A) Wild-type embryo. (B) *knypek* or *trilobite* embryo. (C) *knypek; trilobite* embryos. (D) Immunostaining for Fak protein in a 10-somite *kny;tri* embryo (dorsal view with anterior left). Scale bars, 20 μm.

the newly formed intersomitic furrow (Figs. 6A–6C, white arrows). Fak also becomes concentrated in the basal region of the epitheloid cells in the lateral aspect of the somites (e.g., Fig. 6D, white arrow). As the concentration of Fak protein at the intersomitic furrow demonstrates apical–basal polarity of the epitheloid border cells, we will now refer to these cells as "epithelial border cells." Furthermore, phosphorylated Fak is observed at recently formed somite boundaries (Fig. 6G) and at the borders of cells within the myotomes (Fig. 6H). Both Fak protein and phosphorylated Fak persist at intersomitic furrows (Figs. 6E, 6F, and 6H) throughout myotome formation. These data suggest that Fak functions in the formation and maintenance of intersomitic furrows, and in the formation of the myotome.

Fak Expression at Somite Borders in kny;tri Embryos

The recessive mutations *knypek* (*kny*) and *trilobite* (*tri*) were originally isolated due to their convergent extension defect during gastrulation (Solnica-Krezel *et al.*, 1996). Somites in *kny;tri* double-mutant embryos lack internal mesenchymal cells and are thus comprised of two rows of epithelial border cells that are extended in the mediolateral dimension (Henry *et al.*, 2000). *fak* mRNA is expressed normally in the somites and presomitic mesoderm of *kny*, *tri*, and *kny;tri* mutant embryos (Figs. 7A–7C), indicating that internal cells are not necessary for the initiation of *fak* mRNA expression. Fak protein also becomes concentrated in intersomitic furrows and thus at the basal region of epitheloid border cells of somites in *kny;tri* embryos (Fig. 7D, white arrow). Thus, internal cells are not necessary for the establishment of apical–basal polarity in the epithelial border cells of a forming somite, or for the formation of a boundary between somites.

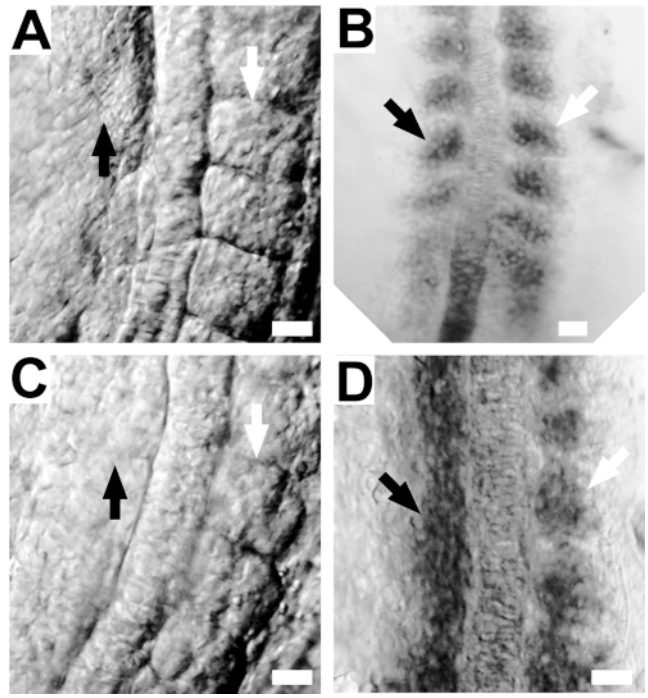

FIG. 8. Notch signaling through *Suppressor of Hairless* is not necessary for normal *fak* mRNA expression; but, ectopic *Suppressor of Hairless* signaling disrupts the normal segmental pattern of *fak* mRNA expression. Black arrows indicate the side of the embryo expressing the construct, while white arrows indicate the control side. (A, C) Embryos viewed with Hoffman optics. (B, D) *In situ* hybridization for *fak* mRNA on the same embryo viewed in bright field. (A, B) A live 10-somite embryo that was injected with a dominant-negative form of *Suppressor of Hairless*, *X-Su(H)1*^{DBM} at the two- to four-cell stage. (C, D) A live 10-somite embryo that was injected with an activated form of *Suppressor of Hairless*, *X-Su(H)1/Ank* at the two- to four-cell stage. Scale bars, 20 μm.

The Role of Notch Signaling in fak Expression in Somites

It has been proposed that Notch signaling may play a role in the initiation of gene expression of genes involved in boundary formation in the presomitic mesoderm (Barrantes *et al.*, 1999). *RBPJ*κ is a mouse homologue of *Drosophila* Suppressor of Hairless, which is a transcription factor that mediates the Notch response (Fortini and Artavanis-Tsakonas, 1994; Jarriault *et al.*, 1995; Tamura *et al.*, 1995). The *Xenopus*-Suppressor of Hairless (X-Su(H)1)^{DBM} construct acts in a dominant-negative manner (Wettstein *et al.*, 1997) and disrupts segmentation in *Xenopus* (Jen *et al.*, 1997). To test whether Notch signaling plays a role in the initiation and/or maintenance of *fak* expression, we injected embryos with *X-Su(H)1*^{DBM} RNA at the 2- to 4-cell stage. Live, injected 8- to 12-somite embryos that had normal somites on one side of the notochord and aberrant or missing somites on the other side of the notochord were photographed, then the same embryo was fixed for *in situ* hybridization to *fak*. In those embryos that were coinjected with GFP mRNA, fluorescence was seen on the affected side and not on the unaffected side (data not shown). Thus, each embryo had an internal control, the nonexpressing side, to which *fak* expression on the opposite side of the embryo could be compared. On the side of the embryo that clearly has no somites (Fig. 8A, left side) *fak* was segmentally expressed (Fig. 8B, $n = 22$ embryos) as in wild-type embryos. Thus, Notch signaling through *Suppressor of Hairless* is not necessary for wild-type segmental *fak* expression.

We also tested whether constitutively activated Notch signaling affects *fak* expression via injection of a constitutively activated form of *X-Su(H)1*, *X-Su(H)1/Ank*. Again, embryos were injected with RNA encoding *X-Su(H)1/Ank* at the 2- to 4-cell stage. At the 8- to 12-somite stage, injected embryos were photographed, and these same embryos were processed for *in situ* hybridization for *fak* expression. Injection of *X-Su(H)1/Ank* perturbs both somite formation and *fak* expression (Figs. 8C and 8D). In embryos

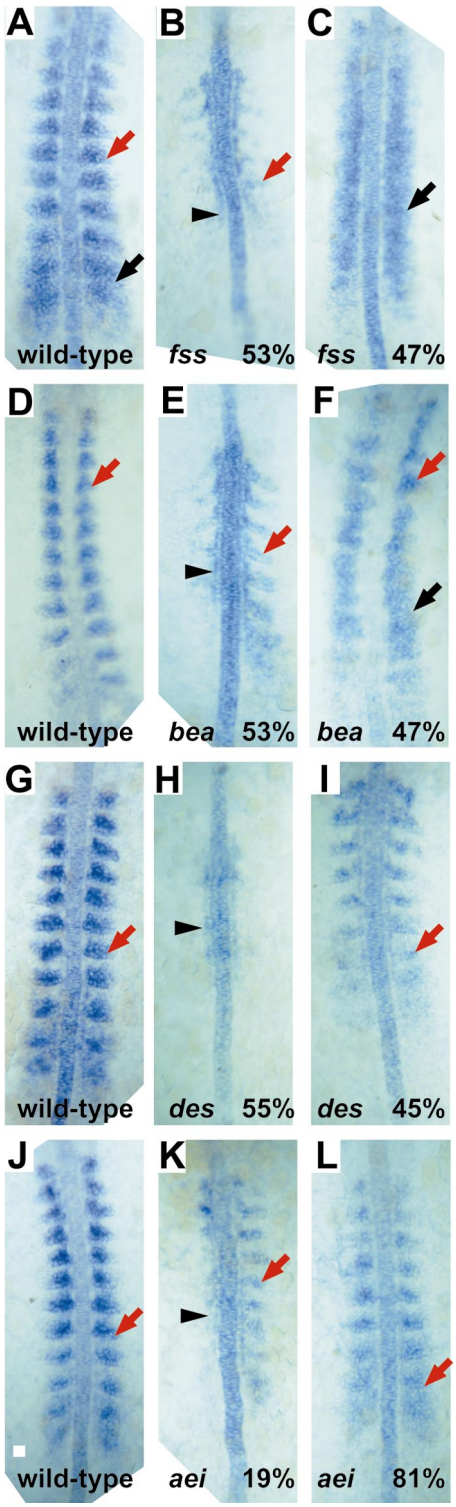

FIG. 9. *fak* mRNA expression is disrupted in the *fss*-type mutants. The percentages denote the percentage of mutant embryos that showed a particular type of *fak* expression. Red arrows denote the last formed somite, black arrowheads denote ectopic *fak* expression in adaxial cells, and black arrows designate nonsegmental *fak* expression. (A, D, G, J) Wild-type embryos at the 10-, 11-, 9-, and 10-somite stages, respectively. All mutant embryos were cultured with their wild-type siblings. (B, C) *fused somite* (*fss*) embryos, which do not form somites. (E, F) *beamter* (*bea*) embryos which form one to four somites. (H, I) *deadly seven* (*des*) embryos which form 5- to 9-somites. (K, L) *after eight/DeltaD* (*aei/DeltaD*) embryos which form 5- to 9-somites. Scale bar in (J), 20 μm.

injected with *X-Su(H)1/Ank*, *fak* expression in the presomitic mesoderm of the two somites that will form next is normal (data not shown). However, the refinement of *fak* expression to a reiterated segmental pattern in the paraxial mesoderm does not take place on the side of the embryo injected with mRNA (Fig. 8D, $n = 23$ embryos). Therefore, ectopic activation of Notch signaling inhibits the mechanism that down-regulates *fak* expression in the anterior region of each formed somite.

The *fused somite* class of mutations is thought to contain mutations in the Notch pathway (Jiang *et al.*, 2000): *after eight*, for example, is a mutation in *DeltaD* (Holley *et al.*, 2000). The formation of somite boundaries and the anterior–posterior patterning of somites are affected in this class of mutations (Durbin *et al.*, 2000; van Eeden *et al.*, 1996). Mutants in this class display an anterior–posterior progression of altered boundaries from no somite boundaries in *fss* embryos, to one to four boundaries in *beamter* (*bea*) embryos, and five to nine boundaries in *deadly seven* (*des*) and *after eight/DeltaD* (*aei/DeltaD*) embryos. Because our *Suppressor of Hairless* experiments indicated a potential role for Notch signaling in *fak* expression, we examined *fak* expression in the *fused somite* class of mutations. Interestingly, each mutant does not have a single, simple affect on *fak* expression: instead, two distinct categories of *fak* expression patterns are seen. The *fused somite* (*fss*)embryos, which have no somites, express either very weak paraxial *fak* in a segmental pattern (Fig. 9B), or nonsegmental *fak* expression similar to the ectopic activation of *Notch* signaling (compare Figs. 9C and 8D). When paraxial *fak* staining is weak, the adaxial cells ectopically express *fak* (compare Figs. 9C and 9A). In *bea* embryos, which form two to five somites, anterior paraxial expression of *fak* either is similar to that in the *fss* embryos that only weakly express *fak*, but the segmental strips are stronger (compare Figs. 9E with 9B) or occur in the anterior four to six somites (compare Figs. 9F with 9C) with nonsegmental *fak* expression posterior. The *des* and *aei* mutants, which have five to nine somites, express *fak* in a segmental pattern in five to nine somites in many embryos (Figs. 9I and 9L). However, the remaining embryos, which also have five to nine somites, have very little or asymmetrical expression of *fak* (Figs. 9H and 9K). Thus, *fak* mRNA expression in many of the *fused somite*-class of embryos is initially normal in the anterior region where somites form, but is absent or nonsegmental in regions in the posterior regions where somites have not formed. However, in the other half of the *bea* and

des embryos, somites formed without normal *fak* expression, indicating that high *fak* expression is not necessary for somite formation.

DISCUSSION

Fak May Play Roles in Intersomitic and Notochord–Somite Boundary Formation

The expression domains of *fak* mRNA, and the subcellular localization of Fak protein and phosphorylated Fak suggest a role for Fak in notochord and somite morphogenesis. FAK protein is localized to intersomitic furrows during *Xenopus*, mouse, and zebrafish development (Hens and DeSimone, 1995; Polte *et al.*, 1994; this paper). We also showed in zebrafish that the mesenchymal-to-epithelial transition of the presumptive border cells in forming somites is accompanied by an accumulation of Fak at the basal region of the cell that abuts the intersomitic furrow. The concentration of Fak at intersomitic furrows increases with time, and is apparent throughout myotome formation. Phosphorylated, and therefore activated, Fak is present at intersomitic furrows, suggesting that one primary role of Fak in zebrafish somites is an adhesive role in the formation and maintenance of somite borders. Our observation that the Fak pool is moderately phosphorylated during somitogenesis, and highly phosphorylated thereafter, supports the hypothesis that cells are stabilizing these adhesive contacts as morphogenesis is completed. This presumptive role for adhesion in somite morphogenesis is consistent with the localization of fibronectin and laminin in the basement membrane of epithelial somite cells in zebrafish (our unpublished observations) and as previously observed in chick embryos (Duband *et al.*, 1987; Krotoski and Bronner-Fraser, 1990). Furthermore, in alpha5 integrin$^{-/-}$ mouse embryos, although the somites condense, they fail to epithelialize at the intersomitic furrows. Thus, alpha5beta1 integrin-mediated adhesion to fibronectin is implicated in the epithelialization of somites (Yang *et al.*, 1999). Therefore, it is likely that Fak associates with integrins and plays a role in the epithelialization, stabilization, and maintenance of the intersomitic furrow through integrin-mediated adhesion.

As in *Xenopus*, an intercalating notochord cell in zebrafish extends a narrow protrusion between two cells, which increases in area as the cell body intercalates (Figs. 4C–4H). In forming notochords, the extension of lamelliform and filoform protrusions between adjacent cells may involve the modulation of adhesions of the leading edge of the intercalating cell (Keller *et al.*, 1989). Fak protein and moderate levels of unstable FAK phosphorylation have been implicated in promoting the turnover of focal adhesions (Ilic *et al.*, 1995, 1996; Schlaepfer *et al.*, 1999; Yu *et al.*, 1998). Thus, it is possible that zebrafish Fak facilitates the modulation of transitory focal plaques as cells intercalate. In addition, because Fak acts through p130^{cas} to facilitate the persistent directional migration of mammalian cells (Gu *et al.*, 1999), it is also possible that one function of Fak in convergence and extension is to promote the directional intercalation of notochord cells. Finally, highly phosphorylated Fak has been linked to an increase in cell adhesion (Yu *et al.*, 1998). Therefore, it is also possible that the plaques of Fak at the leading edge of notochord cells facilitate adhesion when the cell processes encounter the ECM surrounding the notochord and that phosphorylation stabilizes the adhesions. Thus, although abundant in the intercalating notochord cells, Fak is only highly phosphorylated at the periphery. In addition, our observations of striations in phospho-Fak staining in the periphery of the notochord are intriguing in that they suggest a fine structure in the notochordal ECM sheath that may facilitate its anterior–posterior extension. ECM fibers wrapped circumferentially around the notochord would provide strength in the mediolateral dimension while allowing flexibility in the anterior–posterior dimension.

Apical–Basal Polarity May Supplement Anterior–Posterior Identity to Specify Somite Border Formation

One proposed model of somite formation is that the juxtaposition of anterior and posterior cell types induces a somite boundary (Durbin *et al.*, 1998; Evrard *et al.*, 1998; Keynes and Stern, 1988). However, anterior and posterior cell types are also juxtaposed in the middle of a somite, yet a boundary does not form there. As a solution to this quandary, it has been proposed that somites may actually be comprised of three cell states (Meinhardt, 1986). Henry *et al.* (2000) proposed that border formation occurs only at the juxtaposition of basal sides of cells with anterior and posterior identities, thus circumventing the need for a third cell state. The observation that Fak is predominantly localized to the basal region of epithelial border cells in *kny;tri* somites, which lack internal mesenchyme cells, (Fig. 7) is consistent with the hypothesis that apical-basal polarity of cells along with anterior–posterior identity may be sufficient to specify location of a somite boundary.

The Role of Notch Signaling in fak Expression

Several lines of evidence indicate that Notch signaling plays a role in somite formation. Mice mutant for the Notch receptor *Notch 1* have irregular somite boundaries (Conlon *et al.*, 1995), while mice mutant for the Notch ligand *Delta1* show an absence of epithelial intersomitic borders (Hrabe de Angelis *et al.*, 1997). Mice mutant for *RBPJκ*, a transcription factor that mediates the Notch response, form a few small disorganized anterior somites but no posterior somites (Oka *et al.*, 1995). Similarly, injection of a dominant-negative form of the *Xenopus* homologue of *Suppressor of Hairless, X-Su(H)1*^{DBM}, into *Xenopus* oocytes causes disorganized and fewer somites (Jen *et al.*, 1997) and prevents the normal segmental expression of *paraxial protocadherin* (*papc*) in the presomitic mesoderm (Kim *et al.*, 2000). We found, as expected, that

inhibition of Notch signaling through *Su(H)* by injection of *X-Su(H)1*^{DBM} blocks the formation of somite boundaries in zebrafish embryos, and those boundaries that do form are irregular. However, injection of *X-Su(H)1*^{DBM} does not affect either the underlying segmental expression of *fak* mRNA in formed somites or the expression of *fak* in the presomitic mesoderm (Fig. 8).

In *Xenopus*, injection of *X-Su(H)1/Ank*, an activated form of *Su(H)*, perturbs segmental patterning in the presomitic mesoderm as determined by expression analysis of *X-Delta-2* (Jen *et al.*, 1999). We found that injection of *X-Su(H)1/Ank* also blocks the refinement of *fak* expression into a segmental pattern in formed somites and the formation of somite boundaries (Fig. 8). One reason that *fak* mRNA, which is normally down-regulated in the anterior half of the somite, may be expressed throughout the paraxial mesoderm is that the anterior–posterior polarity of the somites may be disrupted by ectopic activation of *Su(H)*. Thus, Notch signaling through *Su(H)* is not necessary for the initiation or maintenance of segmental *fak* expression in the paraxial mesoderm, but ectopic expression of activated *Su(H)* disrupts the refinement of *fak* expression to a segmental pattern in the formed somites. In addition, as the embryos injected with these constructs have either no somite boundaries or aberrant somite boundaries, *fak* expression is not sufficient for normal somite boundary formation.

aei/DeltaD encodes the Notch ligand *Delta D* (Holley *et al.*, 2000). *aei/DeltaD* embryos form five to nine anterior somites (van Eeden *et al.*, 1996), but do not form more posterior somites. *fak* expression in the first eight somites in *DeltaD* mutants suggests that *DeltaD*, like signaling through *Su(H)*, is not necessary for segmental expression of *fak* mRNA in the somites that do form in the anterior of the embryo.

The observation that each fss-type mutant exhibits two categories of *fak* expression patterns indicates that *fak* expression in the *fss*embryos is exquisitely sensitive to the local environment in the paraxial mesoderm. In support of this hypothesis, embryos with different expression patterns on either side of the notochord are frequently observed. The question arises as to how the *bea*, *des*, and *aei* embryos, which exhibit little to no fak mRNA expression, form their anterior somites. Analysis of *fak* expression in the tail of *spt* embryos indicated that there is frequently very little expression, yet somites do form and the Fak protein that is present in the tail of *spt* embryos becomes concentrated at the intersomitic furrows as they form (data not shown). Thus, we would predict that despite the lack of detectable transcription of *fak*, there is some Fak protein (possibly maternally supplied) in the paraxial mesoderm of *bea*, *des*, and *aei* embryos and that this protein does localize to the somite boundaries that do form. Regardless, *fak* mRNA expression is clearly a very sensitive readout of local signaling events in the paraxial mesoderm.

Conclusion

We have cloned zebrafish *fak* and analyzed its expression pattern in wild-type and mutant embryos. Observations of Fak expression in *kny;tri* somites indicate that apical-basal polarity of border cells superimposed upon anterior–posterior identity may be sufficient to specify somite formation. Analysis of embryos injected with dominant-negative Su(H) indicates that Notch signaling is not necessary for *fak* expression. However, the ectopic activation of Notch signaling perturbs the wild-type segmental pattern of *fak* expression. In addition, all of the *fss*-type mutants exhibit disrupted *fak* expression indicating that all of these genes are necessary for normal *fak* expression. An analysis of *fak* mRNA and protein expression and phosphorylation in wild-type embryos implicates Fak in notochord and somite morphogenesis.

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